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Regulation of Amidase Formation in Mutants from *Pseudomonas aeruginosa* PAO Lacking Glutamine Synthetase Activity

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Abstract. The formation of amidase was studied in mutants from *Pseudomonas aeruginosa* PAO lacking glutamine synthetase activity. It appeared that catabolite repression of amidase synthesis by succinate was partially relieved when cellular growth was limited by glutamine. Under these conditions, a correlation between amidase and urease formation was observed. The results suggest that amidase formation in strain PAO is subject to nitrogen control and that glutamine or some compound derived from it mediates the nitrogen repression of amidase.

Key words: Amidase — Nitrogen control — Glutamine synthetase — *Pseudomonas aeruginosa*

Nitrogen control, that is regulation of enzyme formation by the availability of ammonia, has been demonstrated for a number of enzymes in *Pseudomonas aeruginosa*. The synthesis of several proteins that are superfluous when a good nitrogen source is available becomes repressed under these conditions. Thus, during growth with excess ammonia, the cellular levels of urease (Kaltwasser et al. 1972; Janssen et al. 1980), assimilatory nitrate reductase (Sias and Ingraham 1979) and also glutamine synthetase (Janssen et al. 1980) become repressed when compared to nitrogen-limited growth. NADP-dependent glutamate dehydrogenase on the other hand is elevated under ammonia-excess conditions (Brown et al. 1973; Janssen et al. 1980). Under conditions of catabolite repression there is also nitrogen regulation of histidase, urocanase (Lessie and Neidhardt 1967; Potts and Clarke 1976) and enzymes of the arginine catabolic pathway (Mercenier et al. 1980).

The regulation of amidase formation has been elegantly studied by Clarke and coworkers. Amidase formation was found to be regulated by induction, involving positive regulation (Farin and Clarke 1978, Clarke 1980), and catabolite repression, especially by tricarboxylic acid cycle intermediates (Brammar and Clarke 1964). No evidence was found for nitrogen control of amidase synthesis, and repressed levels of amidase were found to be high enough for the generation of ammonia from acetamide in the absence of another nitrogen source (Potts and Clarke 1976).

We have previously isolated mutants from *P. aeruginosa* PAO that lack glutamine synthetase activity (Janssen et al. 1981). The formation of urease and histidase, which were known to be regulated by nitrogen, was no longer repressed in

the mutants by a good carbon and nitrogen source when cellular growth was limited by glutamine. Recently, we have undertaken some measurements on amidase formation in the glutamine synthetase-negative mutants. The results suggest that amidase synthesis in PAO strains is regulated by nitrogen.

Materials and Methods

Organisms

All strains used are derivatives of *Pseudomonas aeruginosa* strain PAO of Dr. B. Holloway. Strain PAO2175 (*met-9020*, *cataI*), which was used as the wild type strain, was isolated by Matsumoto et al. (1978). The isolation of the glutamine synthetase-negative mutants PAO4501 and PAO4506 (formerly designated PAO4001 and PAO4006, respectively) was described before (Janssen et al. 1981). The glutamine synthetase-negative strain PAO4550 was obtained by selecting Ade⁺Leu⁻Gln⁻ recombinants from a cross between PAO12 (*ade-136*, *leu-8*; Pemberton and Holloway 1972) and PAO4501 (R68.45) as the donor. This was accomplished according to the procedures described by Haas and Holloway (1978).

Growth Conditions

Minimal growth medium contained per l: 4.3 g Na₂HPO₄ · 2 H₂O; 2.2 g KH₂PO₄; 100 mg MgSO₄ · 7 H₂O and 1.8 mg FeSO₄ · 7 H₂O. A carbon and nitrogen source was added as indicated. When necessary, L-methionine and L-leucine were added at 0.1 and 1 mM, respectively. The pH after sterilization was 7.0.

For enzyme assays, cells were grown in batch cultures which were inoculated from a washed and diluted suspension of cells pregrown on nutrient broth plus glutamine. Glutamine limitation was achieved in batch cultures by slowly adding glutamine at a constant growth limiting rate (Janssen et al. 1981). Cells were grown overnight aerobically and harvested at an optical density at 600 nm of 0.3–0.6.

Enzyme Assays

Amidase levels were estimated in whole cells by the transferase assay according to the procedure described (Brammar and Clarke 1964; Brammar et al. 1967). Urease, NADP-dependent glutamate dehydrogenase and catechol 1,2-dioxygenase were measured in crude extracts prepared in phosphate buffer by ultrasonic disruption of the cells (Janssen

et al. 1980). The assays for urease and NADP-dependent glutamate dehydrogenase have been described (Janssen et al. 1980; Brown et al. 1973). Catechol 1,2-dioxygenase was measured spectrophotometrically by following the conversion of catechol to *cis-cis*-muconate according to Hegeman (1966). Glutamine synthetase activities were measured in whole cells with the γ -glutamyltransferase assay as described before (Janssen et al. 1980, 1981). One unit of enzyme is defined as the activity that forms 1 μ mol of product per min under the incubation conditions used. Protein was measured according to Lowry et al. (1951), with bovine serum albumin as the standard.

Results

Amidase Formation in the Wild-Type

The cellular level of amidase in *Pseudomonas aeruginosa* PAO1 and PAO2175 was measured after cultivation of the cells on different media (Table 1). It appeared that growth with acetamide as the sole carbon and nitrogen source caused derepression of the enzyme. Catabolite repression was observed when ammonia and a good carbon source such as succinate were added to the growth medium. This confirms the results of Brammar and Clarke (1964) obtained with *P. aeruginosa* strain PAC. With strain PAO2175, however, some elevation of amidase synthesis was observed when ammonia was omitted from the medium and acetamide was the sole nitrogen source (Table 1).

Amidase Formation

in Glutamine Synthetase-Negative Mutants

The regulation of amidase formation was measured in glutamine auxotrophic mutants. Previously, two such strains were described, and they were found to lack glutamine synthetase activity (Janssen et al. 1981). Amidase synthesis in mutant strain PAO4501 was found to be derepressed when the cells were grown in the presence of acetamide, succinate and ammonia under glutamine limitation (Table 1). Apparently, glutamine limitation resulted in a relieve of amidase formation from catabolite repression. The addition of excess glutamine, however, caused a strong repression of amidase synthesis (Table 1). As expected on the basis of earlier results (Janssen et al. 1981) urease levels declined and NADP-dependent glutamate dehydrogenase levels increased in the presence of excess glutamine when compared to glutamine-limited growth.

The glutamine synthetase-negative mutant PAO4506 has a much higher glutamine requirement than strain PAO4501. In PAO4506 glutamine is subject to rapid turnover which makes it impossible to obtain growth under glutamine-excess conditions simply by adding a high amount of glutamine to the growth medium (Janssen et al. 1981). In accordance with this, urease levels are high even when the mutant is grown in a medium containing glutamine and ammonia. The results presented in Table 1 show that also amidase synthesis was not repressed by glutamine in strain PAO4506 in the presence of succinate and ammonia.

Catabolite Repression and Nitrogen Control

It was investigated whether the absence of repression of amidase synthesis by succinate during glutamine-limited

Table 1. Regulation of amidase formation in different strains derived from *Pseudomonas aeruginosa* PAO

Strain	Growth medium ^a	Enzyme activities ^b				
		amidase	GS ₁	GS ₂	GDH	urease
PAO1	ace	3,180	22	26	73	114
PAO2175	ace	2,970	23	32	57	24
PAO2175	ace + suc	1,010	24	22	60	35
PAO2175	ace + suc + amm	270	25	27	67	38
PAO2175	ace + suc + amm + gln _e	40	22	20	40	40
PAO4501	ace + suc + amm + gln _i	1,430	0	0	4	2,350
PAO4501	ace + suc + amm + gln _e	10	0	0	38	45
PAO4506	ace + suc + amm + gln _e	1,060	0	0	2	1,620

^a The growth medium was minimal medium supplemented as indicated: ace = 0.5% acetamide; suc = 1% disodiumsuccinate · 2 H₂O; amm = 0.2% (NH₄)₂SO₄; gln_e = 0.2% glutamine for strains PAO2175 and PAO4501 and 1% glutamine for strain PAO4506; gln_i = glutamine added at a growth-limiting rate.

^b Amidase and glutamine synthetase (GS) activities are expressed in mU/mg dry weight. Glutamine synthetase was measured in the presence of 5 mM Mg²⁺ (GS₁) and with 60 mM Mg²⁺ (GS₂). NADP-dependent glutamate dehydrogenase (GDH) and urease are given in mU/mg protein

growth of the glutamine synthetase-negative mutants was due to poor catabolite repression under these conditions. The occurrence of catabolite repression was tested with catechol 1,2-dioxygenase, an enzyme which is known to be subject to strong repression by succinate in *Pseudomonas* (Ornston 1966). Furthermore, the enzyme is only involved in carbon metabolism, which makes nitrogen control of its synthesis unlikely.

Because strain PAO2175 and its derivatives lack catechol 1,2-dioxygenase, the mutation responsible for the glutamine requirement of strain PAO4501, which is located at about 15 min on the PAO chromosomal map (Janssen et al., manuscript in preparation) was transferred to an appropriate catechol 1,2-dioxygenase-positive recipient. Surprisingly, the resulting strain PAO4550 was found to have the high glutamine requirement characteristic for PAO4506 rather than PAO4501. In a medium containing citrate, ammonia and 0.2% glutamine strain PAO4501 reached a final density of 0.6 mg dry weight/ml, while strains PAO4506 and PAO4550 reached 0.13 mg dry weight/ml. Thus, the mutation that causes the difference in glutamine requirement between strains PAO4501 and PAO4506 is located in strain PAO4501.

With benzoate as the inducer, it was found that succinate caused strong catabolite repression of catechol 1,2-dioxygenase in strain PAO1 (Table 2). When the glutamine synthetase-negative strain PAO4550 was grown in medium containing benzoate and glutamine, catechol 1,2-dioxygenase synthesis was strongly repressed. Addition of succinate to the medium further lowered the level of the enzyme. It appeared that catabolite repression is still functional in a mutant lacking glutamine synthetase activity. Urease levels in strain PAO4550 were high under these conditions, which indicates that the elevation of enzyme levels during glutamine-limited growth is specific for enzymes subject to nitrogen control. When amidase formation in strain PAO4550 was tested, it was found that amidase was not subject to strong catabolite repression by succinate. Both amidase and urease levels

Table 2. Catabolite repression and nitrogen control

Strain	Growth medium ^a	Enzyme activities ^b			
		CO	amidase	GDH	urease
PAO1	benz + amm	810	— ^c	190	280
PAO1	benz + amm + suc	100	—	67	60
PAO4550	benz + gln + amm	30	—	10	1,000
PAO4550	benz + gln + amm + suc	0	—	6	1,210
PAO4550	ace + gln	—	1,750	0	1,560
PAO4550	ace + gln + amm	—	1,500	1	1,550
PAO4550	ace + gln + amm + suc	—	1,920	0	1,850

^a The growth medium contained minimal medium, supplemented as indicated. benz = 0.2% benzoic acid; amm = 0.2% (NH₄)₂SO₄; gln = 1% glutamine; ace = 0.5% acetamide; suc = 1% disodium-succinate · 2 H₂O.

^b Catechol 1,2-dioxygenase (CO), NADP-dependent glutamate dehydrogenase (GDH) and urease activities are given in mU/mg protein. Amidase is expressed in mU/mg dry weight.

^c = not determined

remained high in a medium containing acetamide, glutamine, ammonia and succinate. NADP-dependent glutamate dehydrogenase was negatively correlated with these enzymes.

Discussion

In this paper we describe the regulation of amidase synthesis in strains from *Pseudomonas aeruginosa* PAO. In PAO2175, catabolite repression of amidase synthesis by succinate could be partly overcome by omitting ammonia from the growth medium and using acetamide as sole nitrogen source. In the glutamine synthetase-negative mutant PAO4501, amidase was derepressed during glutamine limitation and the catabolite repression by succinate was relieved. Excess glutamine caused repression of amidase. In strains PAO4506 and PAO4550, which grow under glutamine limitation even when excess glutamine is added to the growth medium (Janssen et al. 1981), amidase was always high. These results show that amidase is regulated in a way similar to histidase, which is described to be subject to nitrogen regulation (Lessie and Neidhardt 1967; Potts and Clarke 1976; Janssen et al. 1981). The lack of repression of amidase in the glutamine synthetase-negative strains was not due to poor catabolite repression. Whereas catechol 1,2-dioxygenase was severely repressed by succinate and glutamine in PAO4550, amidase levels remained high. The levels of amidase in PAO4550 are regulated much more alike urease, which is known to be regulated by nitrogen (Janssen et al. 1981), than to catechol 1,2-dioxygenase, which is regulated by catabolite repression (Ornston 1966; this paper).

P. aeruginosa strain PACIII did not show an escape from catabolite repression of amidase synthesis during nitrogen-limited growth (Potts and Clarke 1976). This could point to differences between PAC and PAO strains with regard to amidase regulation. The formation of amidase was not yet tested in mutants from strain PAC that lack glutamine synthetase activity or have other mutations that affect nitrogen control.

In *P. aeruginosa* PAO, both amidase and urease synthesis are subject to regulation by the availability of ammonia,

which is mediated by glutamine or some compound derived from it (Janssen et al. 1981). Nitrogen control is thus not only involved in the control of the synthesis of enzymes that are involved in the catabolism of potentially biosynthetically valuable compounds. The function of this mechanism is probably mainly the prevention of the formation of unnecessary enzymes, and the strong and selective stimulation of the production of enzymes required under specific nitrogen-limited conditions.

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